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Video Article

Monitoring Protein-RNA Interaction Dynamics in vivo at High Temporal Resolution using χ CRAC

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Abstract

The interaction between RNA-binding proteins (RBPs) and their RNA substrates exhibits fluidity and complexity. Within its lifespan, a single RNA can be bound by many different RBPs that will regulate its production, stability, activity, and degradation. As such, much has been done to understand the dynamics that exist between these two types of molecules. A particularly important breakthrough came with the emergence of 'cross-linking and immunoprecipitation' (CLIP). This technique allowed stringent investigation into which RNAs are bound by a particular RBP. In short, the protein of interest is UV cross-linked to its RNA substrates in vivo, purified under highly stringent conditions, and then the RNAs covalently cross-linked to the protein are converted into cDNA libraries and sequenced. Since its conception, many derivative techniques have been developed in order to make CLIP amenable to particular fields of study. However, cross-linking using ultraviolet light is notoriously inefficient. This results in extended exposure times that make the temporal study of RBP-RNA interactions impossible. To overcome this issue, we recently designed and built much-improved UV irradiation and cell harvesting devices. Using these new tools, we developed a protocol for time-resolved analyses of RBP-RNA interactions in living cells at high temporal resolution: Kinetic Cross-linking and Analysis of cDNAs (χ CRAC). We recently used this technique to study the role of yeast RBPs in nutrient stress adaptation. This manuscript provides a detailed overview of the χ CRAC method and presents recent results obtained with the Nrd1 RBP.

Introduction

RNAs often rely on RBPs to exert their function, which has led to great interest in understanding the dynamics between these molecules. Many RBPs have been identified in a wide variety of organisms. However, it has always been notoriously difficult to study RBP-RNA interactions in vivo. A major breakthrough in studying such interactions came with the emergence of CLIP¹. This method utilizes ultraviolet (UV, 254 nm) irradiation to induce covalent bonds between RBPs and their directly bound RNAs (i.e., zero-distance cross-linking). Subsequently, the RBP of interest is immunopurified under stringent conditions to ensure that only RNAs covalently cross-linked to the proteins are identified. Bound RNAs are then partially digested with RNases and subsequently converted into cDNA libraries for sequencing. The high purification stringency is important as it greatly increases the specificity of protein and RNA recovery, which is also further enhanced through SDS-PAGE purification of the cross-linked ribonucleoprotein (RNP) complex. CLIP and related methods also provide nucleotide resolution insight into the protein binding site, because during the preparation of the sequencing library, amino acids that cross-linked to the RNA frequently terminate the reverse transcriptase or cause the enzyme to introduce mutations at this site^{1,2,3}.

Since its introduction, the original CLIP protocol has produced a remarkable variety of derivative methodologies. A particularly important breakthrough came with the development of HITS-CLIP (or CLIP-seq), which merges high-throughput sequencing with the CLIP approach³. This has since been adopted by all CLIP-based methodologies. iCLIP introduced improvements in the RNase-mediated trimming and adaptor ligation techniques that facilitate more accurate mapping of the RBP binding sites⁴. PAR-CLIP combined 4thio-uridine/uracil labeling with cross-linking at 365 nm, making it possible to map cross-linking sites by analyzing T-C substitutions⁵. CRAC, urea-iCLIP, dCLIP, and uvCLAP introduced denaturing conditions and dual affinity purification steps that further reduce background binding to the affinity resin and further increase the specificity of protein capture^{2,6,7,8,9}. Additionally, CRAC, uvCLAP, and dCLIP introduced tagging the RBP of interest with an affinity tag, thus overcoming the need to generate specific antibodies.

Several optimizations have also been made to expedite the CLIP methodology. The original CLIP protocol utilized radiolabeling of the captured RNAs in order to visualize the RBP-RNA complexes after SDS-PAGE. However, the use of radioactivity can be problematic for laboratories not set up for such work. irCLIP incorporates a fluorophore-coupled adaptor that facilitates visualization through infrared imaging¹⁰ and sCLIP utilizes biotinylation of captured RNAs in order to visualize them through streptavidin-conjugated HRP¹¹. Furthermore, eCLIP completely forgoes RNA labeling; instead the protein is excised based solely on its known size¹². Streptavidin-based purification has also been used to speed up

the process of library preparation in FAST-iCLIP, where a biotinylated 3' adaptor is ligated onto the RNAs and used to enable purification after reverse transcription and circularization¹³. Additional enhancements to the iCLIP protocol also greatly increased the complexity of the libraries⁴.

Finally, CLIP has been modified to enable capture of RBPs from different cellular subcompartments^{14,15}, to visualize newly transcribed RNAs using pulsed induction of photoactivatable ribonucleosides^{5,16,17}, to capture methylated RNAs^{18,19,20}, to examine RNA-RNA interactions^{21,22}, and to map 3' ends^{23,24}.

Despite the great contributions of CLIP-based techniques in aiding our understanding of the interactions between RBPs and RNAs, it has been limited by the inefficiency of UV cross-linking. Although culture cells grown in a monolayer are generally relatively easy to cross-link, this is significantly more challenging in tissues or cells in solution. Tissues can require multiple rounds of UV exposure in order to penetrate to the required cell layers, while microbial cells are often grown in rich mediums that contain aromatic, UV-absorbing compounds²⁵. Indeed, UV irradiation times of up to 30 min have been used to generate sufficient cross-linking between RBPs and their bound RNAs for such samples^{26,27,28}. This extended UV exposure induces stress responses within the cell, such UV-induced DNA damage, which can contaminate the final data in some applications.

The majority of CLIP studies have focused on generating single "snapshots" of specific protein-RNA interactions in a cell. However, protein-RNA interactions are inherently dynamic, particularly when cells are subject to changes in their environment. This can include a sudden reduction in the availability of essential nutrients or rapid changes in temperature. As such, to truly understand the role of an RBP during stress, it is best to perform time-resolved analyses because they can capture the full spectrum of RBP targets during stress and determine at what stage of the stress response the chosen RBP is active. In particular, studies in yeast showed that the first few minutes of adaptation are absolutely crucial for survival and RNA half-lives in bacteria can vary from minutes to seconds^{29,30,31,32,33}. Therefore, such time-resolved analyses should ideally be performed at high temporal resolution. However, the long cross-linking times make the study of early stage adaptive responses particularly challenging.

In order to overcome these issues, we recently developed an improved method that is capable of cross-linking and harvesting cells on minute-long timescales. Our χ CRAC method allows quantitative measurement of dynamic changes in RBP-RNA interactions at previously unwitnessed resolution. Crucial to this method was the development of a novel UV irradiation device³² that reduces the required cross-linking time in yeast and bacteria in solution around 10-fold, effectively freezing RBP-RNA interactions instantaneously. In addition, in order to rapidly harvest the cells after UV irradiation, we developed a vacuum filtration device that can harvest exponentially growing yeast in a 0.5 L culture in around 30 s³². These technological innovations allow the study of RBP-RNA dynamics at minute-scale resolution. Additionally, we also introduced several optimizations to the original CRAC protocol² in order to increase its practicality.

Using χ CRAC, we recently studied the targetome of a yeast nuclear RBP, Nab3, in response to glucose deprivation. In *Saccharomyces cerevisiae*, Nab3 can form a complex with Nrd1, an RBP, and the RNA helicase Sen1 to form the NNS complex. NNS binding to the RNA polymerase and the nascent transcript can trigger transcriptional termination³⁴. This complex is mostly involved in removing cryptic noncoding RNA transcripts but has also been shown to regulate expression of protein-coding genes. The study showed differential targeting of Nab3 to noncoding and coding transcripts after only a minute of stress³². We demonstrated that co-transcriptional termination by Nab3 results in a very transient, pulse-like expression of retrotransposon genes, which would have been difficult to detect using traditional CLIP-based approaches. Additionally, the short UV irradiation times in our UV cross-linker also significantly increased the recovery of short-lived noncoding RNAs³². χ CRAC will likely be a crucial tool in elucidating not only how RBPs shape the response to stress on immediate timescales but also their changing roles during the whole lifecycle of a response. This manuscript provides a detailed overview of all the steps in the χ CRAC protocol. For illustrative purposes, the method was used to study the yeast Nrd1 protein, which is involved in transcriptional termination and RNA decay^{35,36}, and its RNA targetome in response to glucose deprivation across a multitude of timepoints. Finally, we also demonstrate that our UV irradiation unit can rapidly cross-link RBPs to RNA in HeLa cells, making it possible to also perform high-resolution time-resolved analyses in adherent cells.

Protocol

TN150

50 mM Tris pH 7.8

150 mM NaCl

0.1% NP-40

1X protease inhibitor

TN1000

50 mM Tris pH 7.8

1M NaCl

0.1% NP-40

NP-PNK

50 mM Tris-HCl pH 7.8

10 mM MgCl₂

0.1% NP-40

5 mM beta-mercaptoethanol

5 x PNK

250 mM Tris-HCl pH 7.8

50 mM MgCl₂

50 mM beta-mercaptoethanol

WB I

50 mM Tris-HCl pH 7.8

300 mM NaCl

10 mM imidazole

6M guanidine-HCl

0.1% NP-40

5 mM beta-mercaptoethanol

WB II

50 mM Tris-HCl pH 7.8

50 mM NaCl

10 mM imidazole

0.1% NP-40

5 mM beta-mercaptoethanol

Elution buffer

50 mM Tris pH 7.8

50 mM NaCl

250 mM imidazole

0.1% NP-40

5 mM beta-mercaptoethanol

Protease K buffer

50 mM Tris

0.1% NP-40

5 mM β-mercaptoethanol

1% SDS

5 mM EDTA

50 mM NaCl ₂
Mammalian lysis buffer
50 mM Tris-HCl pH 8
100 mM NaCl
0.5% v/v Triton X-100
0.25% w/v Na-deoxycholate
0.1% w/v SDS
5 mM EDTA
1 mM DTT (added fresh)
1X protease inhibitor

Table 1: The buffers required for xCRAC and their compositions.

1. UV cross-linking and lysate production

1. Microorganisms in solution

- Inoculate 3.5 L of the desired medium with yeast from an overnight culture to a starting OD₆₀₀ of 0.05. Grow at 30 °C with continuous shaking at 180 rpm.
- During the growth, prepare other necessary materials.
 - Prepare a container of liquid nitrogen.
 - Prepare 3 L of stress-inducing medium and warm to 30 °C in a water bath.
 - Set up the filter apparatus, turn on the cross-linker (**Figure 2A**) and label 50 mL conical tubes, one for each timepoint.
- Once the cells reach your desired OD₆₀₀, pour 500 mL of cells straight into the cross-linker and UV irradiate with 250 mJ of 254 nm UV. See **Figure 2A** and **Figure 3A** for details on using the crosslinker.
NOTE: The UV irradiation energy must be carefully optimized for each protein of interest. See the Discussion for further details.
- After cross-linking, filter the cells using one of the vacuum filtration devices (**Figure 2B,C**). Roll up the membrane with the filtered cells, place in the t = 0 (time zero) 50 mL conical tube, and flash-freeze in liquid nitrogen.
- Filter the remaining cells over six different filters. Resuspend the collected cells in the 3 L of previously warmed stress-inducing medium by dropping the membranes in the medium and mixing vigorously with a stripette for 50 s. After the 50 s, prepare for taking the t = 1 sample.
- After 1 min, crosslink 500 mL of cells and harvest through filtration as in steps 1.1.3–1.1.4. Repeat after 2, 4, 8, 14, and 20 min, or different timepoints as needed.
- Store the conical tubes containing the cells at -80 °C. Set phosphate buffered saline (PBS) at 4 °C overnight.
- The next day, take each conical tube containing a crosslinked sample and resuspend the cells in 25 mL of cold PBS by shaking vigorously.
- Transfer the cell suspensions to new conical tubes and spin at 4,600 x g, 5 min at 4 °C.
- Pour off the PBS, quickly spin again to collect residual PBS and then decant the remaining liquid with a pipette.
- Calculate the weight of the pellet in the tube by comparing it to an empty tube.
- Add two pellet volumes of ice-cold TN150, 60 µL of DNase 1, and 10 µL of RNase inhibitor. Incubate on ice for 30 min.
 - For example, for 400 mg of cells, add 800 µL of ice-cold TN150.
 - The addition of the DNase is not essential for most soluble proteins but is very important when studying chromatin-bound proteins such as RNA polymerase. Additionally, it reduces the viscosity of bacterial lysates. It is very important to use exactly two pellet volumes of the lysis buffer, or the lysis efficiency can decrease.
- Add three pellet volumes (in mL) of zirconia beads to the cell suspension. For yeast, use 0.5 mm diameter beads and for bacteria use 0.1 mm.
 - For example, for 400 mg of cells, measure out 1.2 mL of zirconia beads in a 1.5 mL tube and add them to the cells resuspended in lysis buffer.
- Vortex the cell suspensions for 1 min, then place on ice for 1 min. Repeat for a total of 5x.
- Add two pellet volumes of TN150 buffer and vortex vigorously to mix.
- Centrifuge the suspension in the conical tube at 4,600 g for 20 min at 4 °C in a benchtop centrifuge.
 - After centrifuging, take a 50 µL sample of the supernatant for future Western blot analysis to examine the whole cell protein expression.
- Transfer the supernatants into 1.5 mL tubes and spin the lysate for 20 min at 20,000 x g at 4 °C, in a microfuge.
 - Alternatively, if using 5 mL tubes, centrifuge at 13,000 x g for 20 min.
 - Following centrifugation, take a 50 µL sample of the supernatant for future Western blot analysis to examine the soluble expression of the protein.
- Proceed to RBP capture (section 2).

2. Cultured adherent cells

1. Seed enough adherent cells in a Petri dish 24 h prior to UV cross-linking so that they can reach 80% confluency the next day. Grow overnight in the desired medium in a cell culture incubator at 37 °C, 5% CO₂.
NOTE: If using quartz Petri dishes, it is beneficial to promote cell adhesion through the treatment of the cultureware with poly-D-lysine (70,000–140,000 wt) and fetal calf serum (FCS) 2.5 h before seeding. Add enough poly-D-lysine to cover the entire growth surface and incubate at room temperature (RT) for 5 min. Next, the quartz Petri dish should be rinsed thoroughly with water and dried in the cell culture incubator for 2 h or until completely dry. Afterwards, add enough FCS to completely cover the growth surface and place in the incubator for at least 30 min. The FCS should be completely removed before seeding cells.
2. Once the cells have reach 80% confluency, remove the media and wash with 15 mL of ice-cold PBS. Next, completely remove all remaining liquid and proceed immediately to the next step.
3. Transfer the Petri dish to the tray for adherent cells (**Figure 3B**) and UV irradiate with 300 mJ of 254 nm UV. See **Figure 2A** and **Figure 3B** for details on using the crosslinker.
NOTE: The UV irradiation energy must be carefully optimized for each protein of interest. See the Discussion for further details.
4. Immediately after cross-linking, place the Petri dish on ice and add 10 mL of ice-cold PBS. Collect cells by scraping and transfer to a 15 mL conical tube. Pellet through centrifugation at 300 x g for 5 min at 4 °C.
5. Remove the PBS and resuspend the cell pellet in 1 mL of ice-cold PBS, and transfer to a 1.5 mL microcentrifuge tube. Pellet cells again by centrifugation for 5 min at 300 x g at 4 °C.
6. Remove the PBS and snap-freeze the cell pellets on dry ice. Store the cell pellets at -80 °C until required.
7. Repeat steps 1.2.3–1.2.6 for each timepoint.
8. Resuspend the cell pellets in 1 mL of lysis buffer and transfer to a 15 mL conical tube. Afterwards, add 1 mL of lysis buffer for a total of 2 mL.
9. Add 5 µL of mammalian RNase inhibitor.
10. Sonicate 5x for 10 s on ice at 10 amp. Wait 30 s between sonication rounds.
11. Calculate the protein concentration of each sample and normalize to the lowest concentration.
12. Transfer 1.98 mL of lysate to a 2 mL tube.
13. Add 10 µL of DNase I and incubate at 37 °C for 5 min with shaking at 1,200 rpm.
14. Centrifuge the lysate at 16,000 x g for 20 min at 4 °C.
 1. After centrifugation, take a 50 µL sample of the supernatant for future Western blot analysis to examine soluble expression of the protein.
15. Proceed to RBP capture (section 2).

2. RBP capture

1. Wash the magnetic anti-FLAG (75 µL of slurry per sample) or IgG agarose (500 µL of slurry per sample) beads 3x with 5 mL of TN150. Resuspend in a final volume of 700 µL of TN150 and add 100 µL of washed beads to seven 15 mL conical tubes.
 1. Store on ice until required.
2. Once the lysates have been clarified, add the supernatant to the tube containing the anti-FLAG/IgG beads.
3. Nutate at 4 °C for 2 h.
NOTE: Some protocols describe overnight incubations with the beads, but this is not recommended, because long incubation times can dramatically reduce the recovery of cross-linked RNAs.

3. Washing the beads and TEV cleavage of the tags

1. Harvest the beads and remove the lysate.
 1. Take a 50 µL of sample of the supernatant for future Western blot analysis to examine the uncaptured protein.
2. Resuspend the beads in ice-cold TN1000 and transfer to a 1.5 mL tube. Wash for 10 min, 4 °C, with nutation. Repeat for a total of three washes.
 1. If using IgG agarose beads, wash with 5 mL of TN1000. If using anti-FLAG beads, use 2 mL.
3. Next, wash the beads 3x with TN150, with the same volume as above.
4. After the third wash, resuspend the beads in 600 µL of TN150.
5. Add 30 U of homemade GST-TEV protease to the bead suspension and rotate for 2 h at RT.
NOTE: Recombinant GST-TEV protease is now also commercially available, but it has not been tested with this protocol.
 1. During the digestion, prepare for the next steps by setting up columns of three 1.5 mL tubes for each sample (i.e. for seven samples, have three rows of seven columns).
 2. To the final row of tubes, add 0.4 g of guanidium hydrochloride, 27 µL of 5M sodium chloride and 3 µL of 2.5 M imidazole (pH = 8). Note that the pH of the imidazole must be 8. This is critical to maintain RNA integrity.
 3. Additionally, wash the required volume of nickel beads in WB I 3x. Use 100 µL of slurry per sample. After the final wash, resuspend the beads in the same original volume of WB I and store on ice.
6. Once the TEV digestion is complete, collect the supernatant using a magnetic rack for anti-FLAG beads or centrifugation for IgG beads, and transfer to the first row of the tubes previously set up.
 1. Take a 50 µL of sample of the TEV eluate for Western blot analysis.
7. Set a thermoblock incubator to 37 °C. To the second row of tubes, add 1 µL of RNase cocktail (1:50 dilution).
8. Take 550 µL of TEV eluate from the first row of tubes and add to the second row (containing the RNase cocktail). Pipette vigorously to ensure mixing.

9. After completing this for the first sample, immediately place the tube into the thermoblock and start a timer. Move on to the subsequent samples, such that each is staggered.
10. Incubate for exactly 5 min. Once completed, remove the first sample from the thermoblock and transfer the solution to the third row of tubes (containing the guanidium hydrochloride powder).
NOTE: A 5 min incubation with a 1:50 dilution of the RNase cocktail is usually suitable for most proteins, but this step will need to be carefully optimized with different incubation times or concentrations for each protein to make sure that the cross-linked RNAs are of the correct size (30–100 nt).
11. Immediately vortex for a couple of seconds at full speed to dissolve the guanidium powder and then move on to the next sample.
12. After all samples have been transferred into the guanidium powder, vortex again to ensure all the powder is fully dissolved.
13. Add 100 μ L of washed nickel beads and rotate overnight at 4 °C. This incubation can be shortened to 2 h.

4. On-bead alkaline phosphatase treatment

1. Set a thermoblock to 37 °C.
2. Place a purification spin column in a 2 mL tube, one for each sample. Transfer the nickel beads to the columns and allow the supernatant to drain through. Afterwards, ensure that all the nickel beads were removed from the 1.5 mL tube by rinsing with WB I and applying to the column.
3. Set up 2 mL tubes, six per sample (one to collect each wash). Keep the outside of the columns dry to maintain flow. Wash the beads 3x with 500 μ L of WB I and then 3x with 500 μ L of NP-PNK.
4. Close the lid of the spin column and briefly spin beads to remove excess buffer.
5. Put the stopper on the column, put columns into 1.5 mL tubes and add 60 μ L of the reaction mixture seen in **Table 2**.

Component	1x	7.5x
5 x PNK buffer	12	90
Alkaline phosphatase	4	30
RNase inhibitor	2	15
H ₂ O	42	315
Final volume	60 μL	450 μL

Table 2: Alkaline phosphatase reaction mixture.

6. Incubate the beads for 1 h at 37 °C.
7. Wash the beads 1x with 500 μ L of WB I to inactivate the alkaline phosphatase and then 3x with 500 μ L of NP-PNK buffer. Make sure to thoroughly rinse the inside of the column with the NP-PNK buffer to remove any traces of guanidium.

5. On-bead ligation of the App-PE linker to the 3' end of the RNA

1. Spin out the remaining buffer and add 60 μ L of the mixture specified in **Table 3** (see **Table 4** for the App-PE sequence) to the columns. Incubate the reaction for 6 h at 25 °C.

Component	1x	7.5x
5 x PNK buffer	12	90
App-PE adapter (100 μ M)	0.6	4.5
T4 RNA ligase 2 truncated K227Q	3	22.5
RNase inhibitor	1.5	11.25
50% PEG 8000	12	90
H ₂ O	30.9	231.75
Final volume	60 μL	450 μL

Table 3: App-PE linker ligation reaction mixture.

Oligonucleotide name	Sequence (5'-3')
L5Aa	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrArArGrCrN-OH
L5Ab	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArUrUrArGrCrN-OH
L5Ac	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrCrGrCrArGrCrN-OH
L5Ad	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrGrCrUrUrArGrCrN-OH
L5Ba	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArGrArGrCrN-OH
L5Bb	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrUrGrArGrCrN-OH
L5Bc	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrArCrUrArGrCrN-OH
L5Bd	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrCrUrCrUrArGrCrN-OH
L5Ca	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrUrArGrCrN-OH
L5Cb	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrGrGrArGrCrN-OH
L5Cc	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArCrUrCrArGrCrN-OH
L5Cd	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrArCrUrUrArGrCrN-OH
L5Da	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrGrUrGrArUrN-OH
L5Db	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrCrArCrUrArN-OH
L5Dc	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrArGrUrGrCrN-OH
L5Dd	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArUrCrArCrGrN-OH
L5Ea	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrArCrUrGrUrN-OH
L5Eb	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrUrGrArCrArN-OH
L5Ec	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrGrUrCrArCrN-OH
L5Ed	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArCrArGrUrGrN-OH
App_PE	App-NAGATCGGAAGAGCACACGTCTG-ddC

Table 4: The sequences of the DNA and RNA adaptors required for ligation onto the 5' and 3' ends of captured RNAs. These were purified through RNase-free HPLC.

- Wash beads 1x with 500 μ L of WB I and 3x with 500 μ L of NP-PNK buffer. Put the column in a new tube and spin out the remaining buffer.

6. On-bead phosphorylation of the 5' ends of the RNA

- Add 80 μ L of the mixture specified in **Table 5** to the columns. Incubate the reaction for 40 min at 37 $^{\circ}$ C.
NOTE: The samples will now be highly radioactive. Thus, all subsequent work should be performed behind a protective screen and waste should be disposed of according to local health and safety rules.

Component	1x	7.5x
5 x PNK buffer	16	120
32 P- γ ATP (10 μ Ci/ μ L)	3	22.5
T4 PNK	3	22.5
H ₂ O	58	435
Final volume	80 μ L	600 μ L

Table 5: Phosphorylation reaction mixture.

- Add 1 μ L of 100 mM ATP and let the reaction proceed for another 20 min. This will make sure that almost all of the 5' ends have phosphates to facilitate ligation of the 5' linker.
- Set up 2 mL tubes, five per sample.
- Wash beads 1x with 500 μ L of WB I and 3x with 500 μ L of NP-PNK buffer. Note that these elutions will be very radioactive and therefore should be disposed of appropriately.
- Move the column to the final tube and spin out the remaining buffer.

7. On-bead ligation of the 5' linker

NOTE: The 5' linkers contain an RNA barcode that is used for identification of each sample after sequencing. Thus, it is absolutely crucial to note which linker is used for which sample.

1. Add 78 μ L of the mixture described in **Table 6** to the columns. Add 2 μ L of 5' adapter (100 μ M; see **Table 4**) to each tube and incubate overnight at 18 °C.

Component	1x	7.5x
5 x PNK buffer	16	120
ATP (10 mM)	8	60
RNase inhibitor	2	15
T4 RNA ligase	4	30
H ₂ O	48	360
Final volume	78 μL	585 μL

Table 6: 5' linker ligation reaction mixture.

2. The following day, wash beads 1x with 500 μ L of WB I and 3x with 500 μ L of WB II and transfer the columns to a new 2 mL tube.

8. Elution, SDS-PAGE, and RNA extraction

1. Set the centrifuge to 4 °C. Prepare two rows of 1.5 mL tubes per sample for elution.
2. Spin out the void volume of the columns with nickel beads with a quick spin. Place the columns in the first row of elution tubes and add 200 μ L of elution buffer. Wait 2 min, then force the buffer through the column with a quick spin.
3. Move the columns to the second row of tubes and repeat step 8.2. Each sample will now have 400 μ L of eluate in total, split over two 1.5 mL tubes.
 1. Take all the eluates and transfer them together to a 5 mL tube. Add 2 μ L of 20 mg/mL of glycogen. Thus, if using seven samples, there will now be 2.8 mL of pooled eluate in the 5 mL tube.
4. Add 100 μ L of trichloroacetic acid (TCA) per sample [e.g. 700 μ L of TCA for 7 samples (2.8 mL of pooled eluate)] to the 5 mL tube, and vortex well for 30 s.
5. Incubate on ice for 20 min.
6. Centrifuge for 30 min at 17,000 x g, 4 °C, in a benchtop centrifuge.
7. Carefully remove the supernatant from the conical tube, checking the pipette with a Geiger counter to ensure the pellet has not been removed accidentally. If it has, return the supernatant to the tube and centrifuge for another 10 min.
NOTE: The supernatant might still be highly radioactive. Make sure to use proper shielding.
8. Fully resuspend the pellet in 2 mL of ice-cold acetone.
9. Centrifuge for 15 min at 17,000 x g, 4 °C.
10. Remove as much of the acetone with a P1000 pipette as possible. Afterwards, briefly spin the tube to gather small droplets of acetone, and then remove with a P10 pipette. Dry for 2 min in a fume hood.
NOTE: The acetone supernatant can still be radioactive. Make sure to use proper shielding.
11. Resuspend the sample in 30 μ L of 1x protein loading buffer. To ensure that the pellet is properly resuspended, check that the vast majority of the radioactivity is now present in the loading buffer and not left in the 1.5 mL tube by removing the solution in a P200 pipette and measuring the activity left in the 1.5 mL tube using a Geiger counter.
12. Heat the sample for 10 min at 65 °C. Load on a 1 mm, 4–12% precast Bis-Tris gel and run for 1.5 h at 125 V in MOPS buffer.
13. After the gel has finished running, open the gel cassette. The gel should be retained on the bottom plate. Dispose of the top.
14. Wrap the gel in cling film and then secure it using tape to the inside of a light-tight cassette. Ensure that the cassette has an amplifying screen to improve the signal.
15. Expose an autoradiographic film to the gel and store the cassette at -80 °C during the exposure. The exposure time will vary between proteins with different cross-linking efficiencies.
 1. When placing the film, there must be a way of realigning it to the cassette in order to cut out the band of interest in the subsequent step. To ensure this, use a fluorescent ruler and also ensure that the gel is at a corner of the cassette, which is then covered by the film also placed in the utmost corner.
NOTE: As a rule of thumb, eluates in loading buffer that give a reading of at least ~250 cps when displayed to a Geiger counter give sufficient signal for an exposure of 3 h. Otherwise, overnight exposure is performed.
16. Develop the film. Cut away the cling film covering the gel but do not move the gel. Otherwise, the image will be offset from the gel.
NOTE: The gel will likely be highly radioactive. Make sure to use proper shielding when cutting out the gel slice.
17. Place the film over the gel and excise the band of interest. Put the gel slice into a 2 mL tube.
18. Smash the gel slice using a P1000 pipette tip and add 600 μ L of proteinase K buffer plus 200 μ g of proteinase K (this protocol uses 10 μ L of a 20 mg/mL proteinase K solution). Incubate for 2 h at 55 °C with vigorous shaking.
19. Afterwards, cut the end of a P1000 tip off with a clean scalpel and transfer the supernatant and gel pieces to a spin column placed in a 2 mL tube.
20. Spin the column for 1 min at 17,000 x g at RT. Collect the flow-through, which contains the radioactive, isolated RNAs.
21. Perform a phenol:chloroform extraction.
 1. Add 50 μ L of 3 M sodium acetate, pH = 5.2, and 500 μ L of phenol:chloroform and vortex well. Spin for 5 min at 17,000 x g. Remove the aqueous top layer and place in a new 1.5 mL tube.
 2. Add 500 μ L of chloroform and vortex vigorously for 10–15 s. Spin for 5 min at 17,000 x g at RT. Remove the aqueous layer and place in a new 1.5 mL tube.
 3. Add 1 μ L of 20 mg/mL glycogen and 1 mL of ice-cold, 96% ethanol. Precipitate for 30 min at -80 °C or overnight at -20 °C.

4. Centrifuge for 30 min at 4 °C, 17,000 x g. Remove the supernatant, add 500 µL of 70% ethanol and centrifuge for 5 min, 4 °C at 17,000 x g. Remove all the ethanol, perform a quick spin to gather residue and remove excess with a P10 pipette.
5. Dry the pellet for ~3 min in a fume hood. Resuspend in 20 µL of DEPC-treated water.

22. Store the RNA at -80 °C overnight or proceed immediately to the reverse transcription step.

9. Reverse transcription

1. Add 2 µL of 10 µM RT oligo (PE_reverse; see **Table 7**) and 4 µL of 5 mM dNTPs to the 20 µL of RNA.

Oligonucleotide name	Sequence (5'-3')
P5 forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
BC1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC7	CAAGCAGAAGACGGCATACGAGATCAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC8	CAAGCAGAAGACGGCATACGAGATTAGCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC9	CAAGCAGAAGACGGCATACGAGATGATCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC10	CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PE_reverse	CAGACGTGTGCTCTTCCGATCT

Table 7: The PCR primers (including the barcode sequences) and the reverse transcription primer.

2. Transfer to a preheated thermoblock at 85 °C for 3 min, then snap-chill on ice for 5 min. Collect the contents of the tube by brief centrifugation and then add 8 µL of 5x reverse transcriptase buffer, 2 µL of 100 mM DTT, and 2 µL of RNase inhibitor.
3. Incubate the mixture at 50 °C for 3 min and then add 2 µL of reverse transcriptase and incubate for 1 h at 50 °C.
4. Inactivate the reverse transcriptase by incubation at 65 °C for 15 min.
5. Transfer the tubes to a preheated thermoblock at 37 °C and leave for 3 min to acclimatize.
6. Add 2 µL of RNase H and incubate for 30 min at 37 °C.
7. Isolate the cDNA using SPRI beads.
 1. Add two volumes of 84 µL of beads. Incubate for 15 min. Put the beads on a magnetic rack and leave for 1 min to harvest the beads.
 2. Remove and dispose of the supernatant and add 200 µL of 70% ethanol. Do not remove the beads from the magnetic rack. Incubate the beads with the ethanol for 30 s.
 3. Remove the ethanol and repeat the wash step. Remove all the residual ethanol using a P10 tip.
 4. Put the beads in a fume hood for 2 min to dry them. Remove the beads from the rack, resuspend them in 12 µL of water, and then put the beads back on the rack. Remove 11 µL of supernatant.
8. Freeze the cDNA at -20 °C or proceed immediately to the PCR step.

10. qPCR reaction

1. Prior to the final PCR for amplification of the cDNAs, a quantitative polymerase chain reaction (qPCR) is performed to identify the optimal number of cycles for amplifying the cDNAs to prevent overamplification of the library.
2. Set up a qPCR reaction on ice according to **Table 8**. See **Table 7** for all primers.

Component	1x
2x qPCR reaction mastermix	5
0.1 µM P5 primer (forward)	0.8
0.1 µM BC primer (reverse)	0.8
cDNA (or water as a negative control)	1
H ₂ O	2.4
Final volume	10 µL

Table 8: qPCR reaction mixture.

3. For proper quantification of the cycles needed for amplification, use three technical replicates for the cDNA and three negative (i.e. water) controls.
4. Seal the plates with optically transparent film and run the qPCR according to the kit manufacturer's instructions.

- Analyze the samples through an absolute quantification method to identify the number of cycles (n) at which the knee of exponential growth is reached (see **Figure 4C** for an example). This number of cycles is then used for the final amplification of the rest of the cDNA.

11. PCR reaction and gel extraction

- Set up the PCR reaction on ice according to **Table 9**. See **Table 7** for all primers.
NOTE: Only 5 μ L of the cDNA library is used.

Component	1x
10 x proof-reading polymerase buffer	5
10 μ M P5 primer (forward)	1
10 μ M BC primer (reverse)	1
5 mM dNTPs	2.5
Proof reading polymerase enzyme	1
cDNA	5
H ₂ O	34.5
Final volume	50 μL

Table 9: PCR reaction mixture.

- Run PCR as follows: 95 °C for 2 min; n cycles of 98 °C for 20 s, 52 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. The number (n) of cycles for amplifying the χ CRAC library is determined by the qPCR described in section 10.
- Add 1 μ L of exonuclease I and incubate at 37 °C for 60 min.
- Clean up the amplified cDNA using SPRI beads as described above using two volumes of beads (i.e. 100 μ L). Elute in 11 μ L.
- Add 3 μ L of 6x loading dye and run on a precast 6% TBE gel at 100 V for 1 h in 1x TBE buffer. Use a ladder appropriate for quantification of short DNA fragments.
- Once finished, remove the gel from the cassette and place in a suitable, liquid-tight container with enough 1x TBE to cover the gel (e.g. ~50 mL). Add an appropriate amount of SYBR safe dye (e.g. for 50 mL, use 5 μ L of a 10,000x dye)
- Allow the gel to stain through gentle mixing for 15 min at RT. Drain the SYBR-containing 1x TBE and replace with clean 1x TBE. Wash the gel for 10 min with gentle shaking at RT.
- Drain the 1x TBE and place the gel in a transparent folder. Cut the folder to an appropriate size.
- Image the gel through an appropriate means such as a phosphorimager. Excise DNA fragments between ~175 bp and ~400 bp. Put the gel slice in a 1.5 mL tube.
- Thoroughly smash the gel slice using a P1000 tip and add 400 μ L of H₂O. Incubate at 37 °C with shaking for 1 h in a thermoblock.
- Freeze the sample on dry ice for 10 min, then place back in the thermoblock at 37 °C with shaking for 1 h.
- Create a filter unit by taking a filter column and inserting two glass microfiber filters inside. Place the unit in a 1.5 mL tube.
- Cut off the end of a P1000 tip with a clean scalpel and uptake the smashed TBE gel suspension, then dispense into the filter unit created in step 11.12. Spin at 17,000 x g for 30 s.
- Add 1 μ L of glycogen to the supernatant, along with 40 μ L of sodium acetate, pH = 5.2, and 1 mL of 96% ethanol. Incubate at -80 °C for 30 min.
- Centrifuge for 30 min at 17,000 x g, 4 °C. Discard the supernatant and wash with 500 μ L of 70% ethanol.
- Spin for 5 min, remove the ethanol entirely and then dry the pellet in a fume hood for 3 min.
- Resuspend in 10 μ L of H₂O and measure the DNA concentration.

Representative Results

To demonstrate the efficacy of the χ CRAC method, a time-course experiment with yeast strains expressing an HTP-tagged Nrd1 protein was performed. A detailed schematic representation describing how the method works is provided in **Figure 1**. Like Nab3, Nrd1 is involved in nuclear RNA decay of a variety of RNA transcripts³⁷. Previous work from the Corden lab suggested that Nrd1 binding to its RNA targets changes significantly when cells are subjected to glucose starvation^{28,38}. As such, cells growing exponentially in medium containing glucose (SD-TRP) were shifted to the same medium without glucose (S-TRP) over a time-course to monitor dynamic changes in Nrd1-RNA interactions. Samples were taken and cross-linked in the Vari-X-linker chamber (**Figure 3A**) before the shift and then after 1, 2, 4, 8, 14, and 20 min. The medium used for cell growth was deliberately deficient in tryptophan to reduce UV absorption by this aromatic amino acid. Note that it is best to use synthetic medium that is filter sterilized as because autoclaving the medium can lead to caramelization of the sugars. This then reduces the cross-linking efficiency.

Figure 4A shows a representative autoradiograph from a χ CRAC experiment. Note that in this example, the samples were not pooled together. Instead, each was run individually on the gel. This is recommended for initial experimental tests to show that the protein cross-links effectively to RNA at all of the tested timepoints. A particularly intense signal was observed at the expected molecular weight of the RBP, representing the protein bound to very short, radiolabeled RNAs not amenable for sequencing. Therefore, the smeary signal above this band, which is the protein crosslinked to longer RNA fragments, was isolated. The fragment was cut from just above the protein band plus around 30 kDa. **Figure 4B** shows an autoradiograph following excision, with the protein cross-linked to short RNAs left in the gel and the previously smeary signal now excised.

After reverse transcription, the cDNA library must be amplified using PCR. However, overamplification of the library must be avoided as this can introduce bias towards sequences preferentially amplified by the polymerase and generate PCR artefacts. Overamplified libraries also contain a large number of duplicate sequences that waste reads on the sequencer. In order to calculate the ideal number of PCR cycles for amplification of the final library, an aliquot of the cDNA was amplified through qPCR using the P5 and BC oligonucleotides. The first cycle at which the library reached peak fluorescence was chosen as the PCR cycle count. **Figure 4C** gives an example of a qPCR from a typical cDNA library, which yielded a peak cycle count of 16. This value was then used for the final χ CRAC PCR. In order to process the sequenced data, we used software previously developed in our lab (pyCRAC) and the corresponding pipeline for analysis of the kinetic CRAC data (Nues et al., 2017; <https://git.ecdf.ed.ac.uk/sgrannem/pycrac>, https://bitbucket.org/sgrann/kinetic_crac_pipeline/src/default/). These open source software tools enable demultiplexing and trimming of the data, removal of PCR duplicates, identification of statistically significant peaks, cluster reads into contiguous sequences, and identify binding motifs³⁹. Further details of how these tools operate are found on their respective webpages.

We also started to develop a χ CRAC protocol for mammalian cells. The majority of mammalian cell lines are grown as a monolayer and the tray in our crosslinker with the UV-permeable bag is not suitable for experiments with adherent cells. To overcome this problem, we developed a stage where users can UV irradiate 1–2 Petri dishes (150 mm diameter and 25 mm in depth) with adherent cells (**Figure 3B**). As a first test, the efficiency of the cross-linker for mammalian cells was measured through cross-linking and capture of stably tagged GFP-RBM7 using anti-GFP antibodies and a traditional CLIP-based purification. As shown in **Figure 5A**, the cross-linker was able to recover protein-RNA complexes from mammalian cells grown as a monolayer using 254 nm UV irradiation at efficiencies comparable to a widely used UV irradiation device. However, standard cell culture plasticware normally used for UV cross-linking experiments is impenetrable to 254 nm UV. Therefore, in our cross-linker the cells would only receive irradiation from the upper bank of UV lamps. To overcome this, we developed a UV-permeable quartz Petri dish for cell growth and cross-linking. Use of the quartz cultureware displayed robust recovery of protein-RNA complexes with as few as 2 s of UV irradiation (**Figure 5B**). When combined with RBP capture methods for mammalian cells such as CLIP technologies, these short cross-linking times are amenable with timecourses to recover spatiotemporal RNA-binding profiles of RBPs in response to genotoxic stresses or rapid depletions of protein factors, or in parallel with transcriptional or cell cycle synchronization.

Figure 6 shows several examples of the Nrd1 data processed by the χ CRAC pipeline. This figure was prepared using the bedgraph files generated by the pipeline and the python GenomeBrowser package (<https://pypi.org/project/GenomeBrowser/1.6.3/>), which we designed to simplify making publication-quality genome browser images of the data. The grey rectangles represent genomic regions that expressed noncoding RNAs, such as the cryptic unstable transcript (CUTs), stable uncharacterized transcripts (SUTs)⁴⁰, and Xrn1-sensitive unstable transcripts (XUTs)⁴¹. The data in **Figure 6** show that Nrd1 binds to many of these noncoding RNA transcripts, consistent with the idea that this protein is involved in degradation of this class of transcripts⁴². **Figure 6A** shows a ~15 kb region on chromosome IV. Here there was a significant increase in binding of Nrd1 to transcripts encoding the high-affinity glucose transporters HXT6 and HXT7, both of which are upregulated during glucose starvation. It is likely that transcription termination by the NNS complex may influence the induction kinetics of these genes during glucose starvation. **Figure 6B** shows an example of Nrd1 cross-linking to the Imd3 transcript, which is known to be regulated by Nab3⁴³. In this case the data demonstrated a significant reduction in binding upon glucose starvation. Previous work showed decreased binding of Nab3 to the Tye7 transcript during glucose starvation⁴⁴. Consistent with this observation, the χ CRAC data suggest that binding of Nrd1 decreased during glucose starvation and Nrd1 cross-linking to Tye7 was at its lowest after 8 min of stress (**Figure 4C**). However, it appears that this effect was only transient, because after 14 min of glucose starvation, Nrd1 binding went back to starting levels.

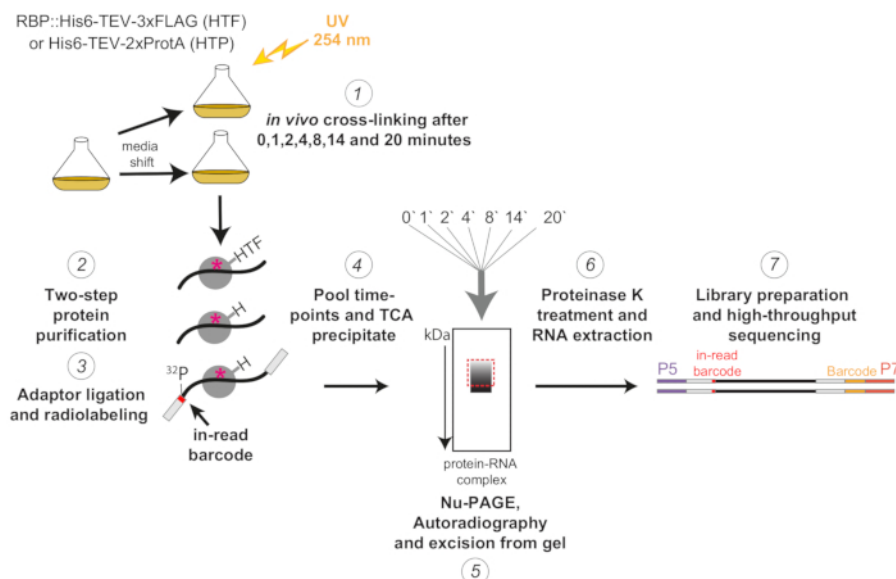


Figure 1: Schematic representation of the xCRAC protocol. Tagged strains were grown until the desired density. RBP indicates RNA-binding protein. Afterwards, a reference sample was taken and cross-linked with 254 nm UV light. The remaining cells were harvested by filtration and then rapidly shifted to the stress-inducing medium. For the xCRAC experiment described here, samples were taken and cross-linked 1, 2, 4, 8, 14, and 20 min after the shift (1). The RBP of interest was then purified using a highly stringent two-step affinity purification (2). Next, the captured cross-linked RNAs were partially digested with RNases, radiolabeled at the 5' end and adapters were ligated onto them (3). The 5' adapters contained unique "in-read" barcode sequences so that the individual samples could be separated bioinformatically after sequencing. The RBP-RNA complexes were then eluted, pooled, and precipitated together (4), resolved by SDS-PAGE and visualized through autoradiography (5). Subsequently, a single gel slice containing the radioactive signal just above the main band, illustrated with dashed red box in the autoradiography image, was cut from the gel (5). The gel slices were treated with protease K and the RNA was subsequently extracted (6), converted into cDNAs, and amplified through PCR (7). The PCR step introduced additional barcodes (yellow block introduced by P7 oligo) so that many libraries could be multiplexed into a single lane. [Please click here to view a larger version of this figure.](#)

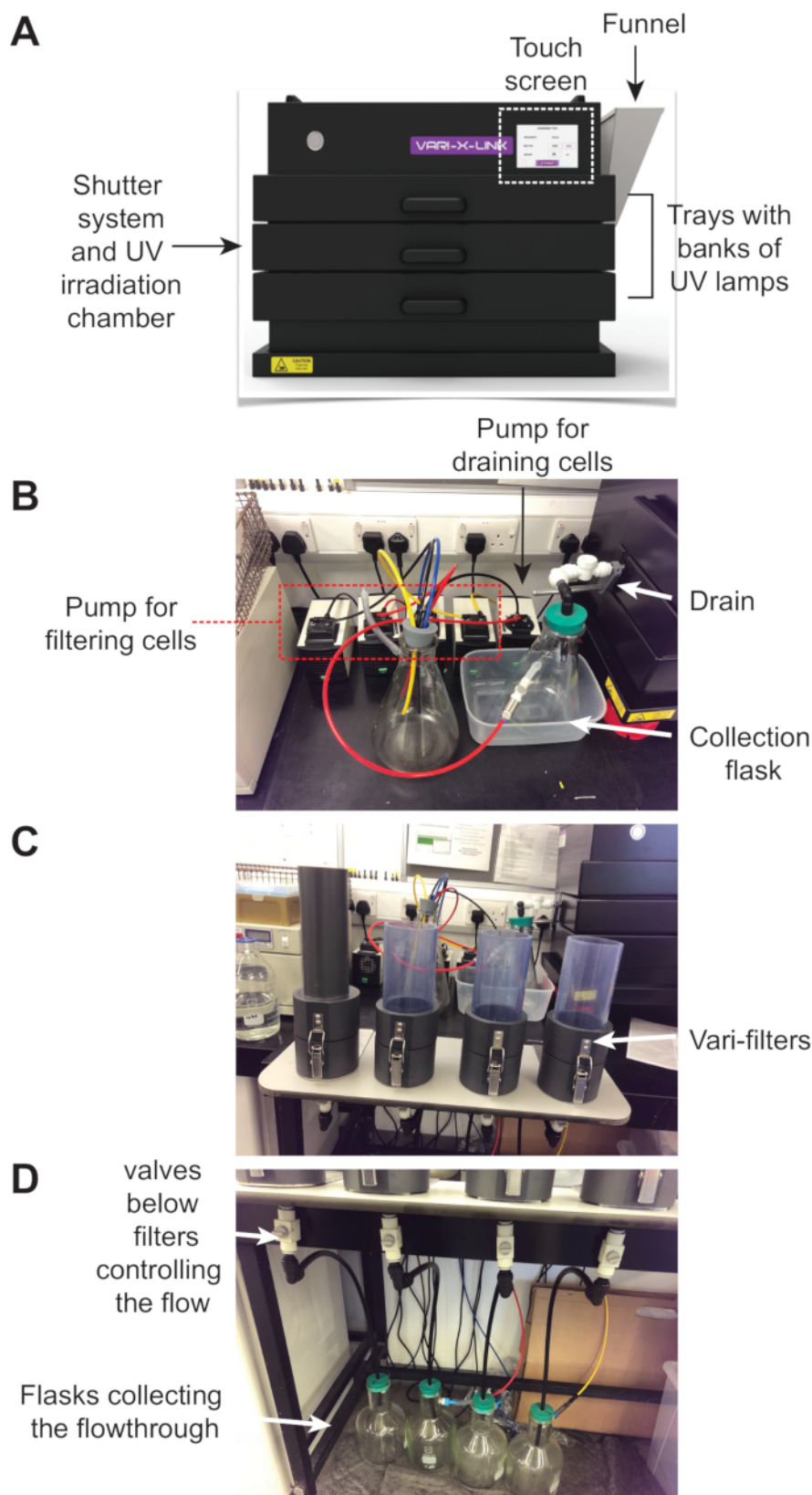


Figure 2: Cross-linking and vacuum filtration. (A) The cross-linker. The cell suspension is poured into a funnel located in the top right of the machine (also see **Figure 3A** for a close-up) and held in a UV-transparent bag located in the middle tray. This bag is flanked by two shutters that remain closed until the user instructs the machine to start the irradiation step. The cells are irradiated with UV light from the trays both above and below. The machine comes supplied with 254 and 365 nm UV lamps, with the latter being applicable for PAR-CLIP experiments. The machine

is operated through a touchscreen panel located on the top right which allows one to control UV dosage or exposure time. **(B)** Following cross-linking, the cells are drained from the left-hand side of the machine. Cell suspensions are recovered through vacuum and drained into a glass flask where they can be subsequently poured into a vacuum filtration device for harvesting. **(C)** Vacuum filtration devices. These are opened and closed via a clip and a filter is inserted between it. Four filtration devices were used in parallel for very short time series to not lose any time as a result of changing filters. **(D)** Following filtration, the media supernatant was drained into flasks for subsequent disposal. Valves were installed below the vacuum filtration devices to maintain the vacuum in the system when the filter is removed. [Please click here to view a larger version of this figure.](#)

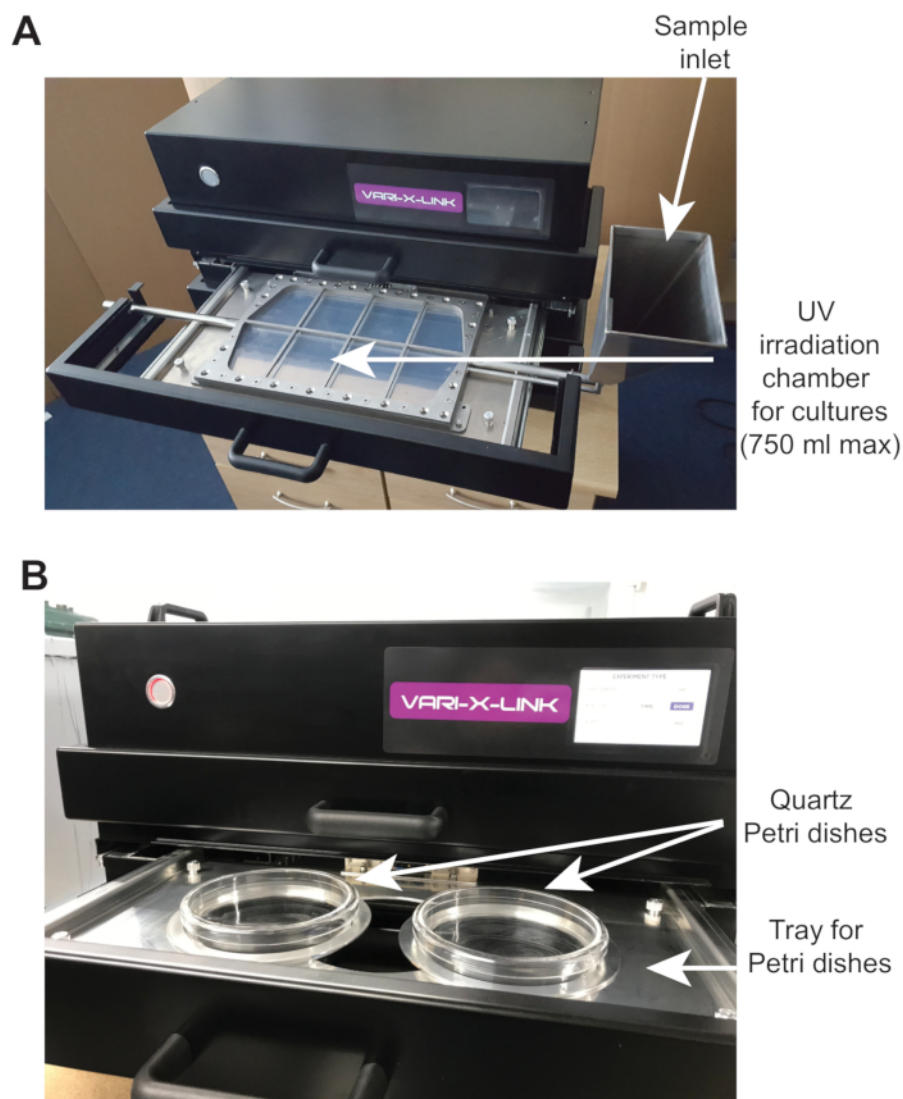


Figure 3: Cross-linking suspended vs. adherent cells. **(A)** The cross-linker with the Vari-X-linker chamber for suspension cells. The cell culture is poured into the sample inlet (funnel) located in the top right of the tray. **(B)** Tray that can hold plastic or quartz Petri dishes for cross-linking adherent cells or small volumes of suspension cells. [Please click here to view a larger version of this figure.](#)

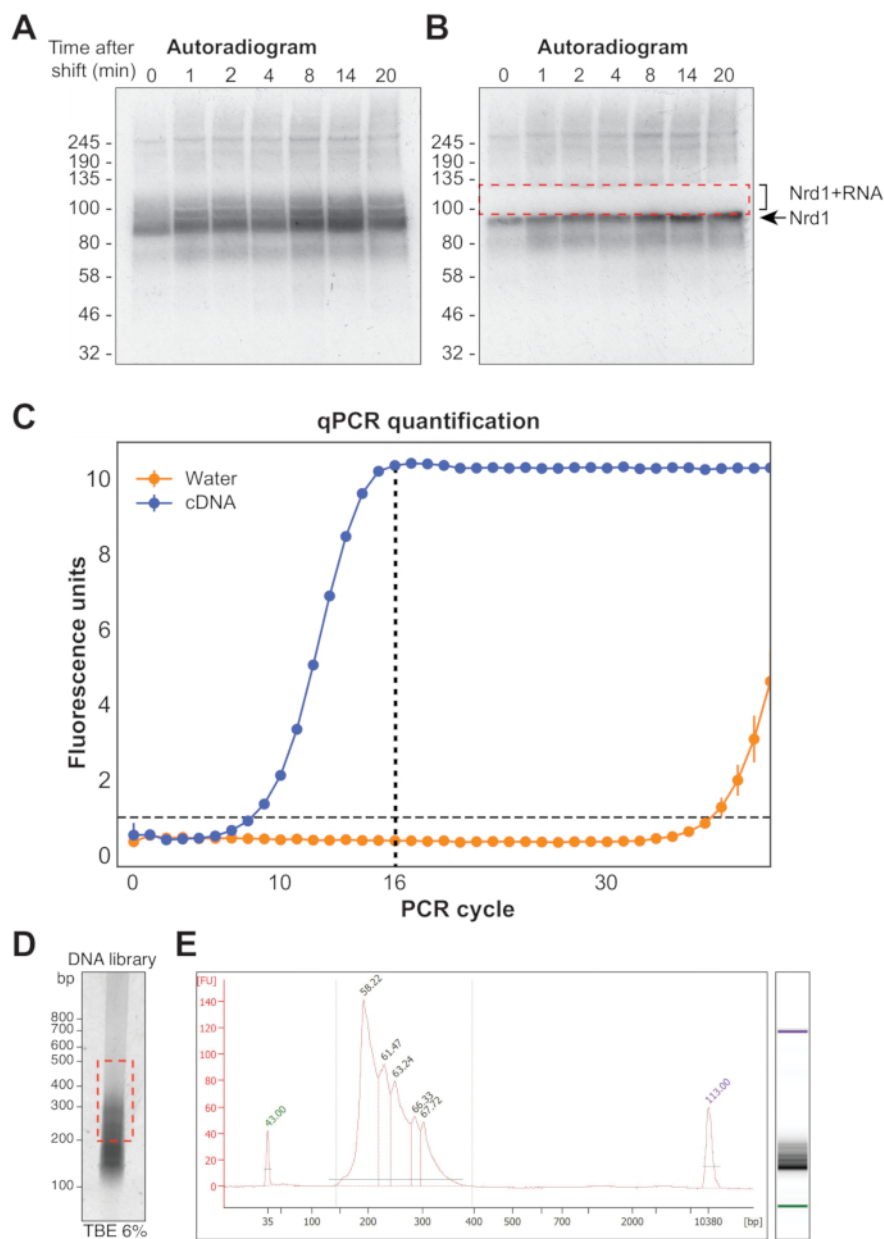


Figure 4: Library preparation. (A) Example of an autoradiogram from a Nrd1-HTP xCRAC experiment. The strong, concentrated signal represents the protein crosslinked to very short RNAs, while the smear above represents the protein cross-linked to RNAs of sufficient length for sequencing. (B) The smear was excised as shown in an autoradiogram taken after gel excision. (C) A representative qPCR from a xCRAC cDNA library. In this example, maximum amplification of the cDNA was reached at 16 cycles. Thus, 16 cycles were used for the final amplification. The error bar represents the standard deviation of three technical qPCR replicates. (D) Example of a phosphorimage from a cDNA library on a 6% TBE gel. (E) cDNA length and quality analysis from a chip-based capillary electrophoresis. [Please click here to view a larger version of this figure.](#)

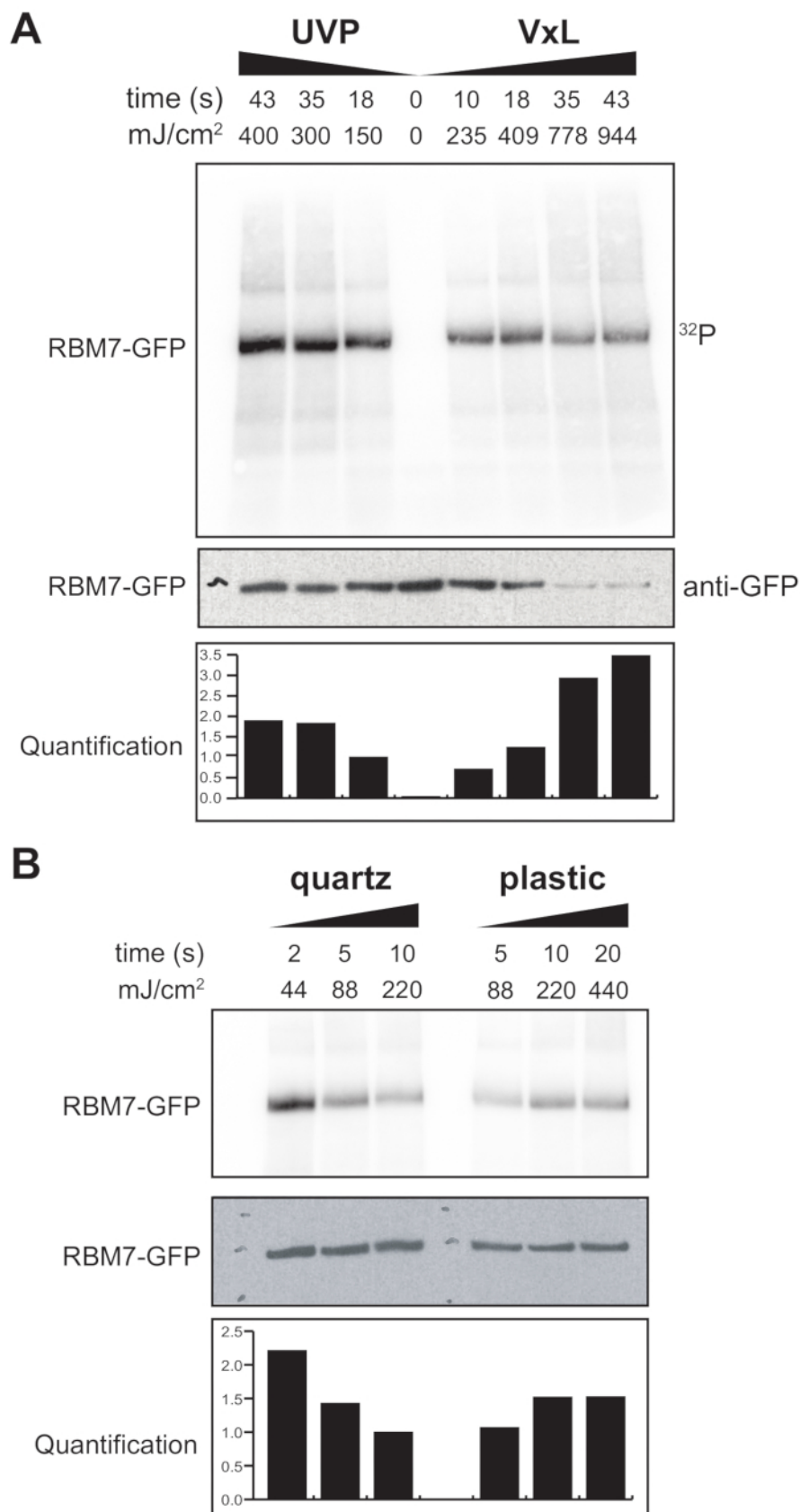


Figure 5: High RNase test iCLIP experiment to test crosslinking in mammalian cells. Shown are autoradiograms from GFP-RBM7 iCLIP experiments which tested the efficiency of RNP recovery across various cross-linking energies. Immunoprecipitations were performed using

anti-GFP antibodies coupled to magnetic beads on cross-linked cells which stably expressed GFP-RBM7. Immunoprecipitates were incubated with high concentrations of RNase I in order to trim associated RNAs to short, uniform lengths. RNPs were visualized by ^{32}P labelling and SDS-PAGE and migrate as a defined band, close to the migration of the non-cross-linked protein. Quantification indicates the results of densitometric analyses of radiolabeled RBM7-RNA signal normalized to the anti-GFP western blot signal. **(A)** Cross-linking time-course of the commonly used UVP cross-linker versus our cross-linker (Vari-X-linker; VxL). **(B)** Cross-linking time-course of our cross-linker on quartz (left) and plastic (right) cultureware. [Please click here to view a larger version of this figure.](#)

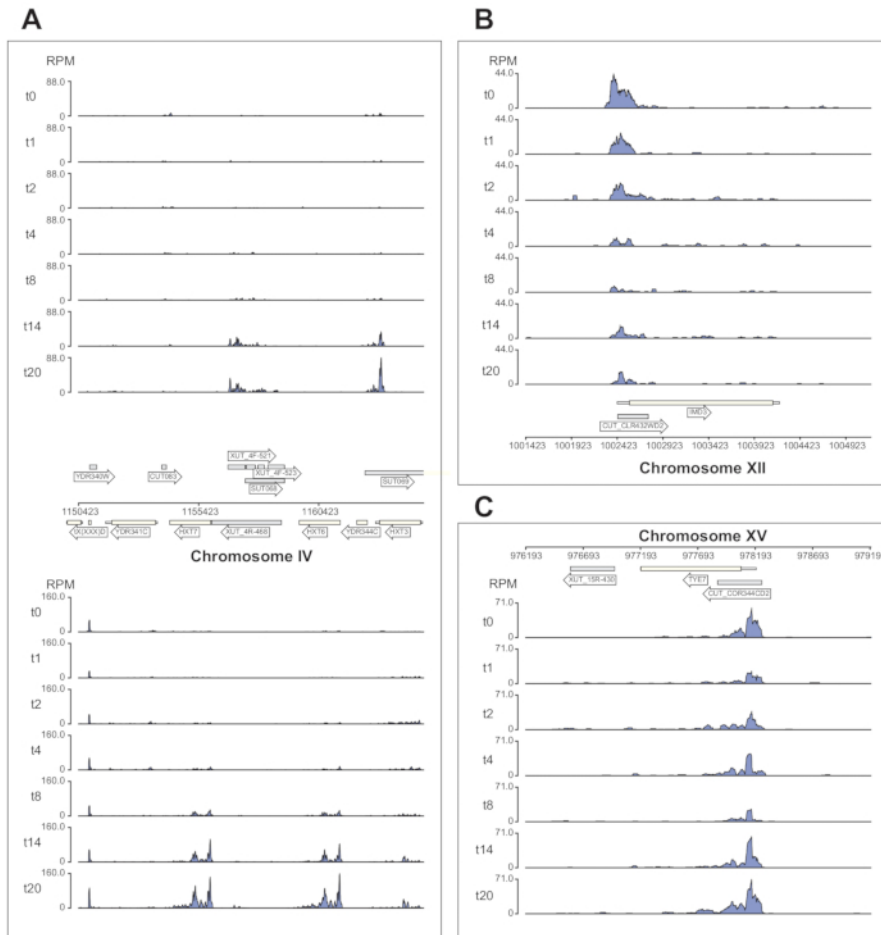


Figure 6: Example genome browser plots showing the power of xCRAC to show differential, temporal binding of Nrd1 to its targets. Each box shows plots for individual genomic regions. The arrows indicate on which strand the genes are encoded (left pointing arrow = minus strand; right pointing arrow = plus strand). The timepoints (min) are indicated by t0, t1, t2, etc on the y-axes of each subplot. Roman numerals indicating the chromosomes and the coordinates are shown. **(A)** Upon glucose deprivation, Nrd1 binds two high-affinity glucose transporters, HXT6 and HXT7, which are both upregulated in this condition. **(B)** Nrd1 is observed to bind to lmd3, an already validated target of Nab3⁴⁴, with reducing intensity following glucose starvation. **(C)** Nrd1 binding of Tye7 exhibits a dynamic and transient nature, decreasing after glucose starvation to a minimum after 8 min of stress. However, binding subsequently returns to basal levels after 14 min. Reads were normalized to "reads per million" (RPM; y-axis). Grey boxes indicate regions encoding noncoding RNAs. [Please click here to view a larger version of this figure.](#)

Discussion

The xCRAC method, combined with the new cross-linking and cell harvesting devices, has great potential because it is applicable to a wide range of model organisms and therefore should be of general interest to the RNA field. There are many areas in which xCRAC can be utilized. For example, the method could be used to measure the hierarchical assembly of proteins into large macromolecular complexes, such as the spliceosome and the ribosome, which often involves dynamic interactions between proteins and RNA molecules. We also now routinely use it to monitor interactions between RNA decay factors and their substrates when cells are subjected to diverse kind of stresses. This enables us to determine at what stage of the adaptive response these factors are most active, what substrates they bind to, and how dynamic these interactions are. Such data should enable researchers to determine the relative contribution of each factor in adaptation to environmental changes.

xCRAC uses dual affinity purification tags (HTF or HTP) to purify the protein under highly stringent and denaturing conditions. This ensures that the copurified RNA is highly enriched for RNAs that were covalently cross-linked to the protein of interest. However, relying on affinity tags has disadvantages. For example, the tag could interfere with protein function, which could give a distorted readout of its RNA-binding interactome. Additionally, for some model organisms it may not always be possible to utilize tags because the genetic tools to integrate DNA fragments

into the genome or to transform expression plasmids are not yet available. However, it is straightforward to alter some parts of the χ CRAC protocol to make it compatible with CLIP-based protocols that rely on antibodies for purification of the RBP. Indeed, this study showed that it is possible to combine iCLIP-based purifications with our crosslinker. We are now in the process of developing CLIP protocols to study the temporal association of human RNA-binding proteins with nascent RNA transcripts.

When performing χ CRAC on a new protein, the UV exposure must be optimized in order to induce maximal cross-linking. This is important because high UV exposures can reduce the recovery of RNA during the purification step. Cells expressing the recombinant RBP were exposed to various UV doses, 100 mJ/cm², 250 mJ/cm², 500 mJ/cm², and 1 J/cm². The RNPs were then captured and the RNAs were fragmented and radiolabeled. Afterwards, the RNPs were resolved by SDS-PAGE and an autoradiogram was taken in order to deduce which exposure gave the most intense signal (i.e. the maximal cross-linking).

Once the experimental conditions are optimized, several control experiments are recommended when performing χ CRAC. First, a UV irradiated, untagged sample can be used to monitor background binding to the purification beads. Second, when applying χ CRAC during a shift experiment, a second time series where the cells are shifted back into the original medium enables investigation into whether the filtration of the cells itself induces changes in RNA levels or protein-RNA interactions.

As mentioned in the Introduction, numerous recently published papers suggest a number of optimizations to the CLIP protocol. This includes the use of fluorescently labeled adapters for detecting the protein-RNA complex through infrared scanning¹⁰ as well as optimizations to various nucleic acid purification and size selection steps shown to increase the complexity of the resulting libraries^{12,45}. We are currently implementing some of these improvements to further refine the χ CRAC protocol. The protocol presented here already contains a number of improvements to the original CRAC and χ CRAC protocols that increase the complexity of the data. For example, previously, after resolving the cross-linked, radioactive protein-RNA complexes on SDS-PAGE gels, they were transferred to a nitrocellulose membrane and the cross-linked RNA was isolated from the blot. However, the transfer of the RNP and subsequent RNA extraction can be very inefficient, particularly when dealing with large RBPs such as RNA polymerase subunits. This can result in a significant reduction in the recovery of the cross-linked RNA. In the current protocol, the cross-linked RNA is extracted directly from SDS-PAGE gel slices as illustrated in **Figure 1**. This increased the recovery of cross-linked RNAs. Additionally, after PCR amplification of the cDNAs the product was originally resolved on 3%, low melting temperature agarose gels, and then 175–300 bp PCR products were extracted from the gel. However, these gels can be easily overloaded, resulting in very poor separation of the DNA. Replacing agarose gels with precast TBE gels resulted in more consistent size separation and better recovery of PCR products.

Disclosures

A. Langford and W. Worboys are affiliated with UVO3, a commercial company. They had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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